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Identification of a Signaling Network in Lateral Nucleus of Amygdala Important for Inhibiting Memory Specifically Related to Learned Fear

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Summary

We identified the *Grp* gene, encoding gastrin-releasing peptide, as being highly expressed both in the lateral nucleus of the amygdala, the nucleus where associations for Pavlovian learned fear are formed, and in the regions that convey fearful auditory information to the lateral nucleus. Moreover, we found that GRP receptor (GRPR) is expressed in GABAergic interneurons of the lateral nucleus. GRP excites these interneurons and increases their inhibition of principal neurons. GRPR-deficient mice showed decreased inhibition of principal neurons by the interneurons, enhanced long-term potentiation (LTP), and greater and more persistent long-term fear memory. By contrast, these mice performed normally in hippocampus-dependent Morris maze. These experiments provide genetic evidence that GRP and its neural circuitry operate as a negative feedback regulating fear and establish a causal relationship between *Grpr* gene expression, LTP, and amygdala-dependent memory for fear.

Introduction

Fear is a basic, evolutionally conserved, emotion, which triggers a set of defensive mechanisms for adapting to threatening events that is essential for survival. A key component of the neural circuitry of fear—both innate and learned—in humans and in simpler vertebrate experimental animals is the amygdala, a well-defined subcortical nuclear group (Davis and Whalen, 2001; LeDoux, 2000).

The memory of learned fear can be assessed quantitatively using a Pavlovian fear-conditioning paradigm (Fanselow and LeDoux, 1999; Kapp et al., 1992). During fear conditioning, an initially neutral conditioned stimulus (CS) acquires biological significance by becoming associated, following a few pairing trials, with an aversive unconditioned stimulus (US). After learning this association, an animal responds to the previously neutral CS with a set of defensive behavioral responses, which includes freezing, increased heart rate, and startle. The CS can be unimodal, involving only a single cue or modality such as a tone, light, smell, or touch. Alternatively, it can be multimodal, involving several sensory modalities such as a context. Unimodal (cued) fear conditioning requires the amygdala but not the hippocampus. By contrast, multimodal (contextual) fear conditioning depends on both the hippocampus and the amygdala.

The lateral nucleus is the input region within the amygdala, where the association of learned information about CS and US occurs during auditory fear conditioning. The sensory information that mediates the CS—the auditory tone—reaches the lateral nucleus by way of two neural pathways, both of which are essential for learned fear (Romanski and LeDoux, 1992). One pathway, the direct thalamo-amygdala pathway, originates in the medial geniculate nucleus (MGm) and in the posterior intralaminar nucleus (PIN) of the thalamus. The second pathway, the indirect cortico-amygdala pathway, extends from the auditory thalamus to the auditory cortex (TE3 area) and includes a further projection that relays the processed auditory information from the cortex to the lateral amygdala. After these two inputs are processed in the lateral nucleus, the signal is distributed to other amygdaloid nuclei (Pitkanen et al., 1997), including the central nucleus of the amygdala (CeA), which projects in turn to areas in the brainstem that control autonomic (heart rate) and somatic motor centers (freezing) involved in the expression of fear.

Anatomical tracing and lesion studies first demonstrated the importance of the lateral nucleus for fear conditioning. Subsequent physiological experiments showed that learning produces prolonged synaptic modification in both of the inputs to the lateral nucleus: the thalamo-amygdala pathway (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997) and the cortico-amygdala pathway (Tsvetkov et al., 2002). These synaptic modifications, which accompany behavioral learned fear, are mechanistically similar to LTP induced artificially by electrical stimulation in tissue slices of the amygdala. By providing a direct causal link between slice LTP and memory storage, these studies establish the amygdala as perhaps the simplest and the best model system in the mammalian brain for analyzing the cellular and molecular mechanisms of memory storage.

In contrast to the detailed cellular physiological information that is becoming available, the molecular machinery that underlies synaptic plasticity in amygdala-dependent learned fear is largely unknown. Toward this

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end, we have combined molecular biology, mouse genetics, physiology, and behavior to analyze amygdala-based learned fear. We first identified two genes highly enriched in the lateral nucleus of the amygdala: the gastrin-related peptide (GRP) and oncoprotein 18 (Op18)/Stathmin. We focused on GRP because it is presumably released as a cotransmitter with glutamate in pyramidal cells of the lateral nucleus and its receptor (GRPR) has been pinpointed by Monaco and his colleagues as a candidate in autism (Ishikawa-Brush et al., 1997). Our data suggests that, when released by activity, GRP acts on and excites inhibitory interneurons by activating GRPR on their cell surface. Activation of GRPR in these interneurons in turn leads to an increase in the level of tonic GABAergic inhibition in the principal neurons.

Using amygdala slices from GRPR knockout mice, we next found that the tonic inhibition is markedly reduced and that LTP is enhanced. Consistent with this finding, these mice have enhanced and prolonged long-term memory for fear to both auditory and contextual cues, suggesting that the GRP signaling pathway serves as an inhibitory feedback constraint on learned fear.

Results

Isolation of Genes Specifically Expressed in the Lateral Nucleus of the Amygdala

As an initial step in characterizing the molecular mechanisms involved in learned fear, we searched for genes enriched in the amygdala and, in particular, in the lateral nucleus (Figure 1A). To this end, we focused on pyramidal projection neurons because these cells form the majority of the constituent neurons in the cortex-like nuclei of the amygdala to which the lateral nucleus belongs and they transmit the CS and US information during fear learning. We isolated neurons using acute dissociation, which preserves their processes and allows cell identification based on neuronal morphology under the microscope (Yu and Shinnick-Gallagher, 1997). Similarly, pyramidal neurons were isolated from the anterior dorsal CA1 subregion of the hippocampus, which was chosen for the comparison during cDNA library screening because this region may be less involved in learned fear as opposed to the ventral hippocampus (Bast et al., 2001). We first used two rounds of representation difference analysis (RDA) to enrich the lateral nucleus cDNA probe against the CA1 cDNA sequences. After differential screening of cDNA library derived from single pyramidal amygdala neuron with probes from the lateral nucleus and the CA1 region, we analyzed candidate clones for gene expression pattern using RNA in situ hybridization. We found two genes, *Grp* and *Oncoprotein 18* (*Op18*)/*Stathmin* expressed in the lateral nucleus of the amygdala that had low or no expression in the CA1 region of the hippocampus (Figures 1B and 2A). Interestingly, these two genes are also expressed in the accessory basal nucleus (AB) of the amygdala, but are absent in the basal lateral nucleus (BLA) that is located between the lateral nucleus of the amygdala and AB.

Both the *Grp* and *Op18*/*Stathmin* sequences originated from the screening of the same cell, which we identified as glutamatergic pyramidal neuron based on its shape during acute dissociation under the microscope and later by hybridizing its cDNA library with different neuronal and glial markers (data not shown) and by subsequent characterization of the sequences comprising this cDNA library. This cDNA library (that contained the *Grp* and *Op18*/*Stathmin* sequences) was positive for neurofilament-L (NF-L, neuronal marker) and it was negative for glial fibrilary acidic protein (GFAP, glial marker) and glutamic acid decarboxilase (GAD, interneuronal marker). In addition, we isolated from this library a cDNA that corresponds to the zinc transporter-3 (ZnT-3) gene, a specific marker for zinc-containing subgroup of glutamatergic neurons, highly enriched in the limbic system and the lateral nucleus of the amygdala (reviewed in Frederickson et al., 2000).

GRP Is Expressed in the Lateral Nucleus of the Amygdala and in the Regions Sending Synaptic Projections to the Lateral Nucleus

Using in situ hybridization, we next found that the *Grp* gene is highly enriched in the lateral nucleus of the amygdala (Figure 2A), and more specifically, in its dorsal and medial subnuclei. In addition, we observed strong expression in the medial, ventral, and dorsal subdivisions of the medial geniculate body (MGm, MGv, and MGd), the posterior intralaminar nucleus (PIN) of the auditory thalamus, the TE3 subregion of the auditory cortex, and the perirhinal cortex (PRh, Figure 2B). All of these regions are afferently connected with the lateral nucleus of the amygdala and provide auditory inputs to the lateral nucleus of the amygdala during fear learning (Pitkanen et al., 1997) suggesting that this peptide is involved in auditory cued fear conditioning. For example, MGm and PIN directly project auditory information to the lateral nucleus of the amygdala and to TE3. Area TE3 of the cortex in turn projects to the lateral nucleus of the amygdala (LeDoux, 2000). The ventral subiculum (VS), another structure where the *Grp* is localized, also provides a strong input to the medial division of the lateral nucleus of the amygdala as well as to BLA and AB. PRh is reciprocally connected with the lateral nucleus of the amygdala and is capable of sending either cued or contextual signals. GRP is also expressed in the ventral dentate gyrus. However, a connection between the lateral nucleus of the amygdala or AB and the dentate gyrus is not well documented.

GRPR Is Expressed in Inhibitory Interneurons

GRP is a peptide neurotransmitter that is selectively recognized by a seven transmembrane domain receptor (GRPR) coupled to Gαq-protein (Hellmich et al., 1999). Having shown that GRP is expressed by principal cells in the lateral nucleus of the amygdala, we were curious to know what types of cells express GRPR. To identify the neurons within the lateral nucleus of the amygdala that express GRPR, we performed colocalization studies using dual fluorescent in situ hybridization for *Grpr* RNA and immunohistochemistry with antibodies against interneuron-specific marker, glutamic acid decarboxylase (GAD67 form, Figure 3A). We found that the *Grpr* RNA was expressed selectively in inhibitory GABAergic interneurons. However, GRPR was present only in a subpopulation of GAD-positive interneurons, which suggests that the lateral nucleus of the amygdala contains

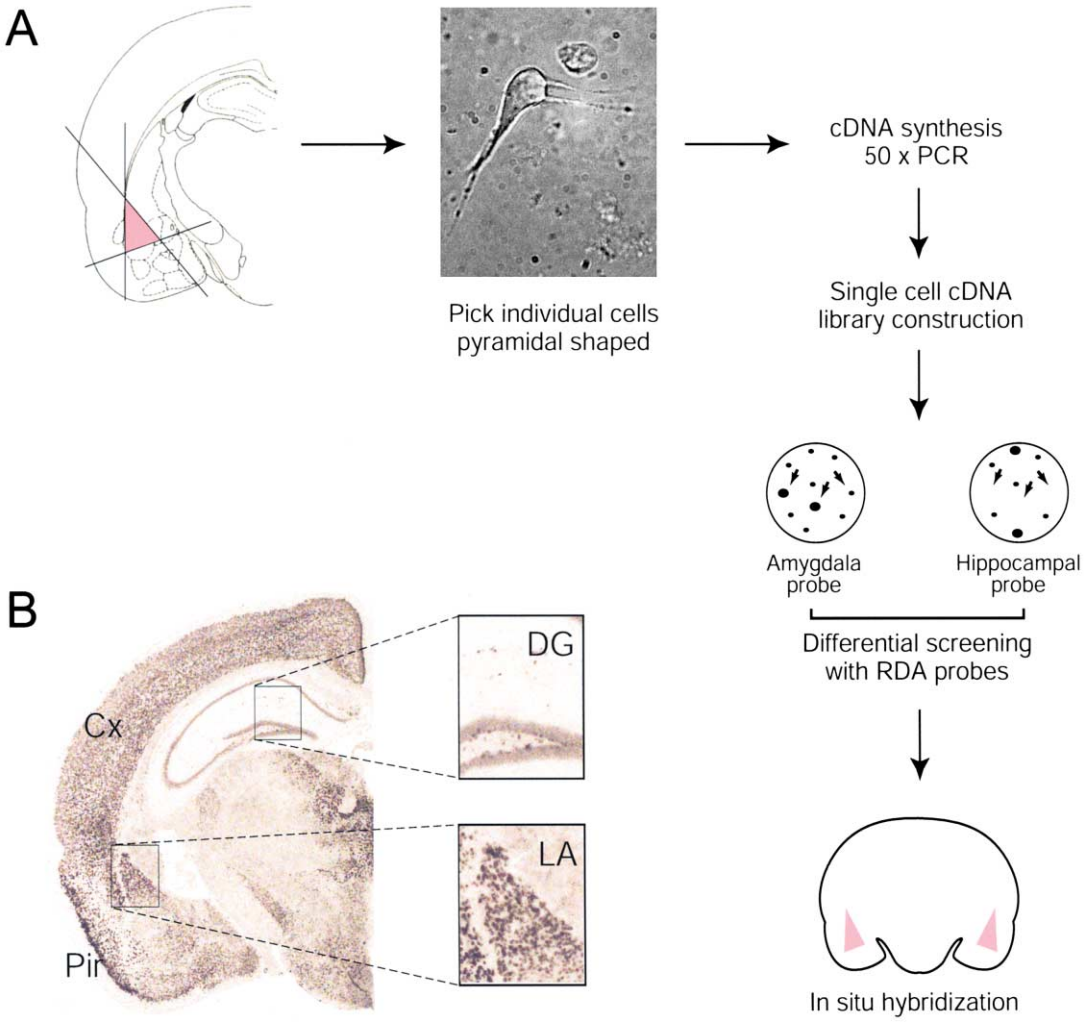


Figure 1. Strategy for Isolating Amygdala-Enriched Genes

(A) Scheme of the differential screening of single cell cDNA libraries from amygdala neurons (with a representative neuron after acute dissociation of the rat amygdala).

(B) *Op18/Stathmin* RNA in situ hybridization on a coronal section of mouse brain. Insets show strong expression in the lateral nucleus of the amygdala and weak expression in the hippocampus.

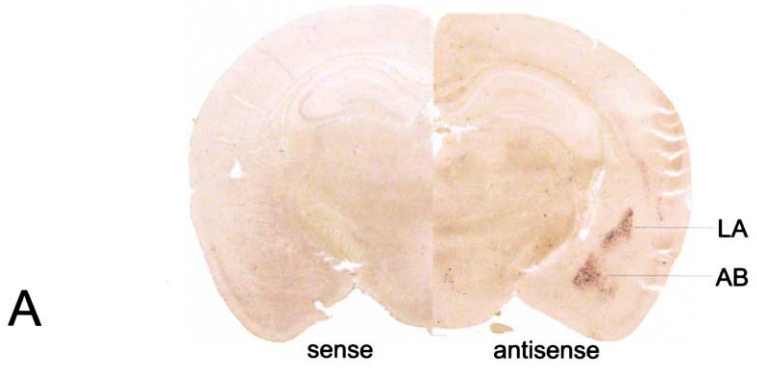
various groups of interneurons subserving different functions.

Physiological, tract-tracing, and immunocytochemical studies have shown that afferent signals converging onto the lateral nucleus of the amygdala are regulated locally in the dorsolateral division by inhibitory interneurons (Woodson et al., 2000). The afferent glutamatergic projections to the amygdala synapse on both principal cells and GABAergic inhibitory interneurons (Mahanty and Sah, 1998). The inhibitory interneurons in turn send feedback inhibitory projections to pyramidal neurons. These feedback and feedforward GABAergic inputs are thought to determine how the excitatory inputs to the principal cells involved in fear learning are processed and conveyed along neural pathways in the amygdala (Wang et al., 2001). The observed pattern of the *Grp* and *Grpr* genes expression (see Figures 2 and 3) suggested to us that GRPR exerts a functional role in modulating the balance between excitation and inhibition in the local neuronal networks related to learned fear.

GRP Appears to Excite GABAergic Inhibitory Interneurons in the Lateral Nucleus of the Amygdala that Functionally Express GRPR

To test whether activation of the GRP receptors on the GABAergic interneurons in the lateral nucleus of the amygdala by the release of GRP from principal cells can change the level of tonic inhibition in the principal cell, we carried out whole-cell recordings from visually identified pyramidal neurons in mice. We identified pyramidal neurons based on their appearance and their ability to demonstrate spike frequency adaptation to the prolonged depolarizing current injection (Tsvetkov et al., 2002).

We recorded spontaneous inhibitory postsynaptic currents (sIPSCs) in the pyramidal neurons having blocked the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor-mediated responses (Figure 4) with CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, 20 μM). To increase the inhibitory signals, we inverted the inhibitory currents so that they had an inward direction by dialyzing the postsynaptic cells with



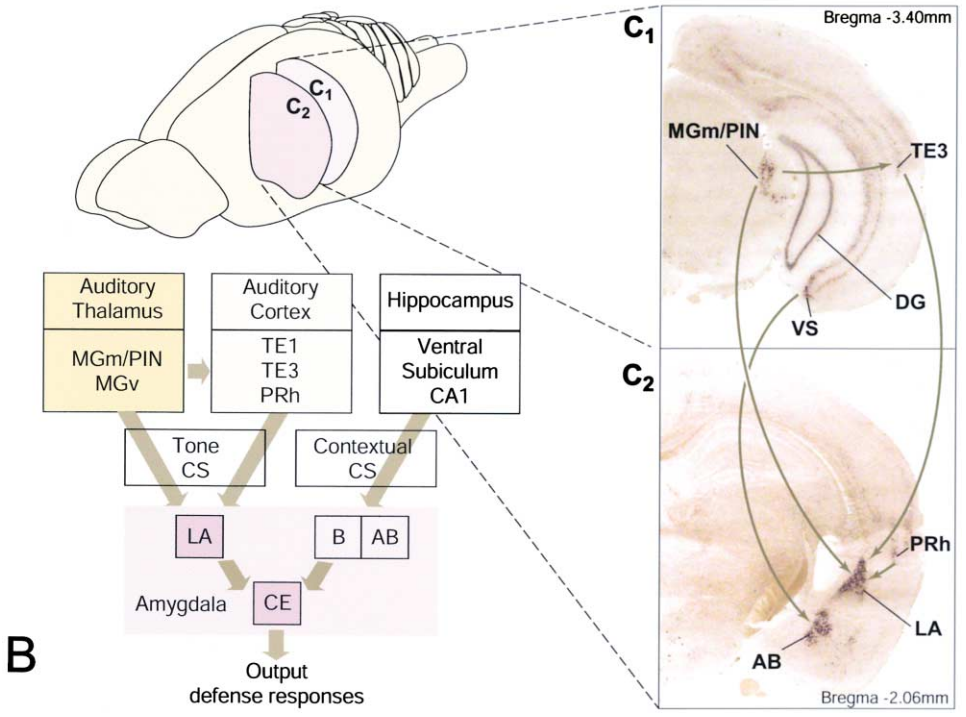


Figure 2. The *Grp* Gene Is Specifically Expressed in the Lateral Nucleus/AB of the Amygdala and in the Cued and Contextual CS Pathways to the Amygdala

(A) In situ hybridization of the *Grp* gene in the lateral nucleus of the amygdala and AB with sense (left) and antisense (right) RNA probes.

(B_1) Mouse brain showing the location of coronal sections C_1 and C_2.

(B_2) Major areas that send auditory and contextual information to the amygdala obtained from tract-tracing studies.

(C_{1-2}) RNA in situ hybridization shows expression of the *Grp* gene in the areas shown in B_2 diagram.

a chloride-based intrapipette solution. Consistent with the notion that the sIPSCs are mediated by the $GABA_A$ receptors, these currents were completely blocked (Figures $4A_3$ and $4B_2$) by γ-aminobutyric acid-A ($GABA_A$) receptor antagonist, picrotoxin (50 μM, n = 10), at a holding potential of −70 mV.

In the absence of a $GABA_A$ receptor blockade, application of GRP (200 nM) led to a significant increase in the frequency of sIPSCs in the soma of the principal cells of wild-type mice (baseline: 5.23 ± 0.68 Hz; GRP: 10.12 ± 1.0 Hz; n = 17 cells, obtained from 5 control mice; significant difference, paired t test, $t = 4.99$, $P < 0.0002$; Figures 4A–4C). Therefore, we think that the increase in frequency of GABA sIPSCs was likely due to excitation of the interneurons by GRP leading to an increase in the firing of action potentials in GABAergic interneurons. We further supported this by blocking the effects of the agonist by applying a Na^+ channel blocker tetrodotoxin (TTX, 1 μM, n = 7; Figure $4A_2$). These findings in the lateral amygdala are consistent with previous work in the hippocampus, where bombesin-like neuropeptides (including GRP) elicited a marked increase in the frequency of $GABA_A$ receptor-mediated IPSCs recorded in CA1 pyramidal neurons (Lee et al., 1999) mediated by depolarization and induced repetitive firing of GABAergic interneurons in the stratum oriens.

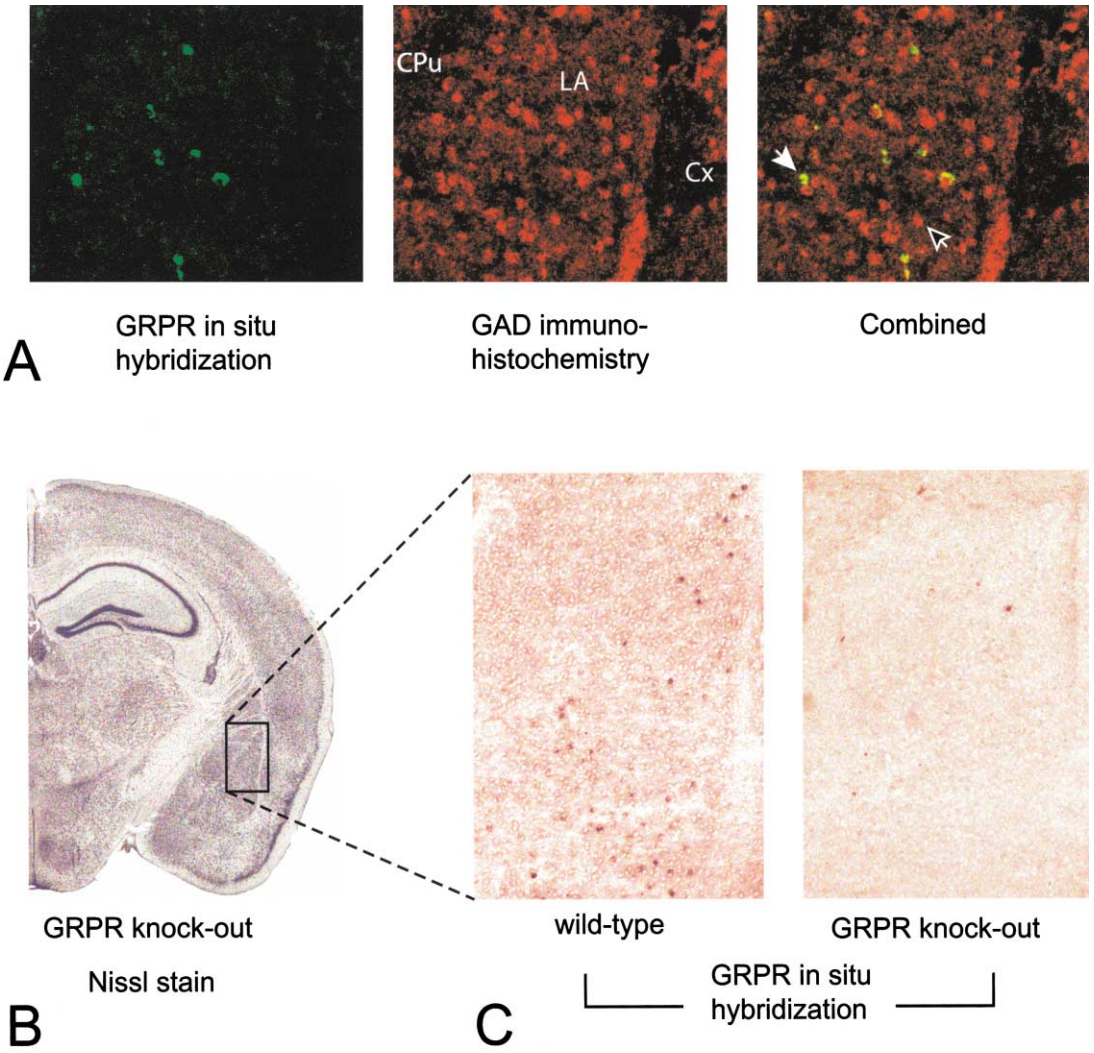


Figure 3. Expression of the *Grpr* Gene in the Amygdala

(A) The *Grpr* gene is expressed by interneurons. Left image, fluorescent in situ hybridization for *Grpr* RNA. Middle image, immunohistochemistry for interneuron marker, glutamic acid decarboxylase (GAD). Right image, *Grpr* and GAD images combined show colocalization of *Grpr* in a subset of interneurons. White arrow, example of an interneuron positive both for *Grpr* and GAD. Black and white arrow, a GAD-positive interneuron that does not express *Grpr*. CPu, caudate putamen; Cx, cortex.

(B) Gross anatomy in the amygdala and in the rest of the brain is normal in GRPR knockout mice (Nissl staining).

(C) In situ hybridization showing *Grpr* expression in the amygdala of wild-type mice (left image). *Grpr* RNA is absent in the amygdala of GRPR knockout mice (right image).

We specifically linked the observed effect of the bath-applied GRP to the activation of GRPR. Bath application of a specific antagonist of GRPR ([D-Phe^6,Des-Met^{14}]-bombesin-(6-14)ethyl amide; 3 μM; Lee et al., 1999) blocked the effect of GRP on the frequency of sIPSCs (Figures 4A$_1$ and 4B$_1$; baseline: 5.15 $\pm$ 0.91 Hz; GRP: 10.37 $\pm$ 1.2 Hz; antagonist of GRPR: 5.72 $\pm$ 1.1 Hz; n = 6 cells). The difference in the frequency of sIPSCs in the baseline conditions and after the GRPR antagonist application was not statistically significant (paired t test, t = 1.21, P = 0.3), suggesting that the bombesin antagonist fully abolished the GRP-induced increase in the frequency of the sIPSCs.

Knockout of GRPR Eliminates Tonic Inhibition

To obtain independent evidence that GRP induces enhancement of GABAergic tonic inhibition due to activation of GRP receptors localized on interneurons, we turned to mice in which the gene for GRPR was knocked out. These mutant mice were littermates of the control mice we have studied to this point. GRPR knockout mice do not show any obvious developmental anatomical abnormalities throughout their body or their brain (Hampton et al., 1998 and Figure 3B). Immunohistochemistry on brain sections of these mice with interneuron-specific antibodies (parvalbumin, calretinin, and calbindin) revealed no differences between knockout mice and wild-type controls (data not shown). However, in situ hybridization revealed that the GABAergic interneurons in the knockout mice were lacking GRPR (Figure 3C). Consistent with these findings, we found in the mutants that the GRP-mediated negative control of the excitatory synaptic inputs to principal cells in the lateral nucleus was lacking. In slices from mice in which the

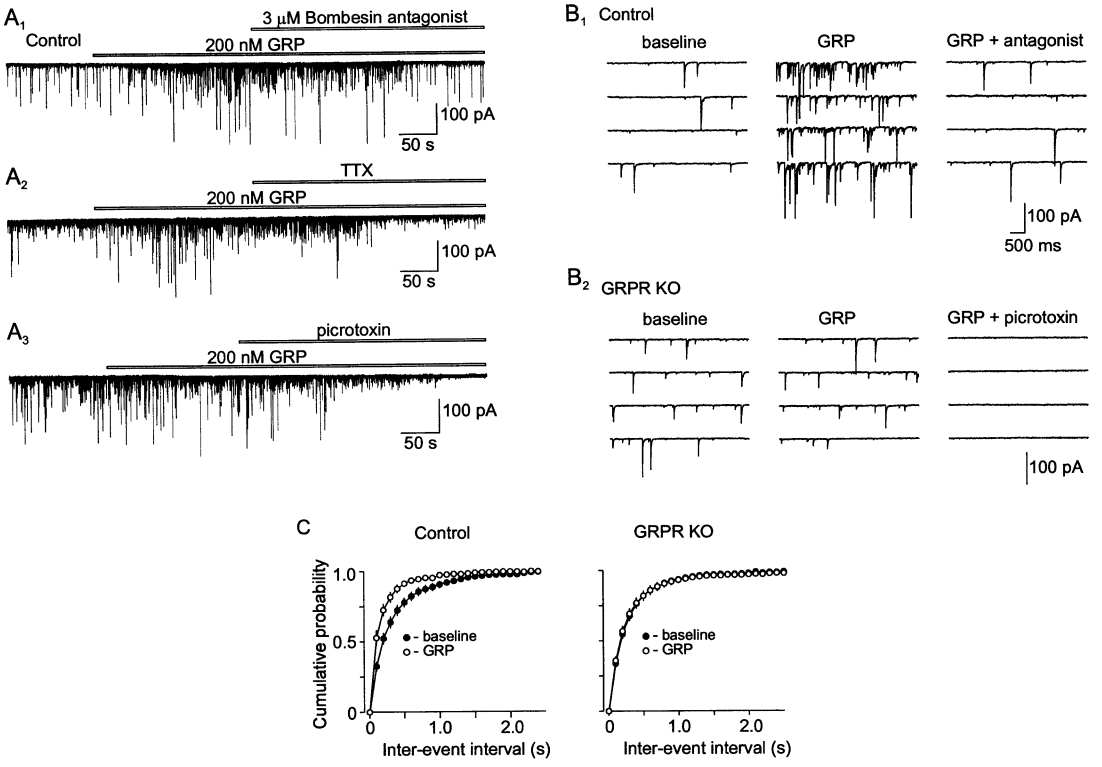


Figure 4. GRP Receptors Are Functionally Expressed in Interneurons of the Lateral Nucleus of the Amygdala

(A_1) Bath application of GRP (200 nM) increased frequency of sIPSCs in a pyramidal cell from a control mouse. The effect was blocked by 3 μM bombesin antagonist (n = 6), thus suggesting that the GRP-induced enhancement of GABAergic tonic inhibition was specifically linked to the activation of the GRP receptors.

(A_2) Effect of GRP on the frequency of sIPSCs is TTX-sensitive, and thus is dependent on action potential firing in interneurons.

(A_3) GRP failed to increase the frequency of the picrotoxin-sensitive sIPSCs in GRPR knockout mice.

(B_1) Representative sIPSCs recorded in a pyramidal cell from a control mouse at a holding potential of −70 mV under baseline conditions (left), during GRP application (center), and after the GRPR antagonist was added (right).

(B_2) Representative sIPSCs recorded in a pyramidal neuron from GRPR knockout mouse under baseline conditions (left), during GRP application (center), and after picrotoxin was added (right).

(C) Cumulative amplitude histograms of sIPSCs recorded under baseline conditions (filled symbols) and after GRP was applied (open symbols) in slices from control (left) and GRPR knockout mice.

Grpr gene was ablated, bath-applied GRP failed to increase the frequency of sIPSCs (200 nM; baseline: 5.06 ± 0.58 Hz; GRP: 5.64 ± 0.67 Hz; n = 23 cells, obtained from 6 GRPR knockout mice; no significant difference: paired t test, t = 1.04, P = 0.31; Figures $4A_3$, $4B_2$, and 4C). These results suggest that GRP receptors are functionally expressed in the lateral nucleus of wild-type mice and that activation of the GRP receptors on these interneurons was responsible for the dramatic increase in the level of tonic GABA inhibition observed in the principal neurons in the lateral nucleus.

LTP in the Cortico-Amygdala Pathway Is Enhanced in GRPR-Knockout Mice

Our recent findings indicate that LTP of the synaptic connections in the neural circuit of learned fear is an essential cellular mechanism contributing to the acquisition of memory for fear (Tsvetkov et al. 2002; see also McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). Studies of different brain regions, including the hippocampus (Steele and Mauk, 1999), the cortex (Trepel and Racine, 2000), and the amygdala (Rammes et al., 2000), indicate that modulation of principal cells by GABA-mediated inhibition can play an important role in the induction of LTP. We therefore asked: does removal of GRPR in the inhibitory interneurons affect LTP in slices of the lateral nucleus of the amygdala?

We induced LTP of the compound glutamatergic EPSCs at the cortico-amygdala synapses by pairing postsynaptic depolarization from a holding potential of −70 mV to +30 mV with 80 presynaptic stimuli delivered to the fibers in the external capsule (Huang and Kandel, 1998; Mahanty and Sah, 1998; Weisskopf and LeDoux, 1999) at a frequency of 2 Hz (Figures 5A–5B). We measured LTP with the K-gluconate containing intrapipette solution, without picrotoxin in the bath (see Experimental Procedures). Under these experimental conditions, the peak amplitude of the evoked EPSC was solely determined by activation of the AMPA glutamate receptors. The contribution of the $GABA_A$ receptor-mediated component to the EPSC was negligible at such holding potential since it was very close to the reversal potential (E_r) of $GABA_A$ IPSC (−67 ± 3 mV, n = 6; Figure 5C). This induction protocol was used because, as we have shown previously, it consistently produces robust

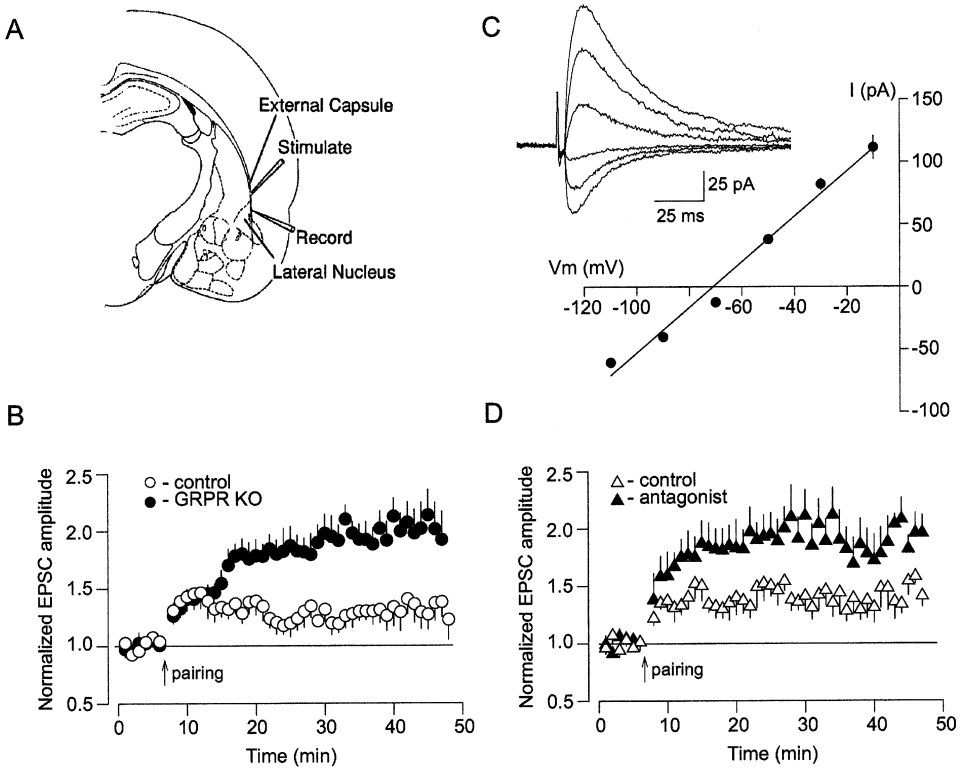


Figure 5. Pairing-Induced LTP Is Enhanced in GRPR Knockout Mice

(A) A schematic representation of a brain slice containing the amygdala that shows position of the recording and stimulation pipettes.

(B) LTP of whole-cell EPSCs recorded in the lateral amygdala neuron in response to the cortical input stimulation in slices from control (open symbols) or GRPR knockout (filled symbols) mice. For induction of LTP, the lateral amygdala neuron was held at +30 mV, and 80 presynaptic stimuli were delivered at 2 Hz to the external capsule fibers (arrow).

(C) Current-voltage plot of the $GABA_A$ receptor IPSCs at holding potentials of −110 mV to −10 mV. Reversal potential of the IPSC mediated by the $GABA_A$ receptors was −71 mV. Synaptic currents were recorded in the presence of the AMPA receptor antagonist CNQX (20 μM) and NMDA receptor antagonist D-APV (50 μM). Inset shows $GABA_A$ receptor IPSCs recorded at holding potentials of −110 mV to −10 mV. Traces are averages of 10 IPSCs recorded at each holding potential.

(D) Pairing-induced LTP of whole-cell EPSCs recorded in the lateral amygdala in wild-type mice under control conditions (open symbols) and in the presence of the bombesin antagonist (3 μM, filled symbols).

LTP (Tsvetkov et al., 2002). We have deliberately chosen to depolarize a postsynaptic cell to a more positive membrane potential during the induction period, than in some previous studies, to allow a maximal activation of L-type Ca^{2+} channels (e.g., Mermelstein et al., 2000), as they were shown to take part in the induction process (Tsvetkov et al., 2002; Weisskopf et al., 1999). Keeping Ca^{2+} influx through L-type Ca^{2+} channels at a relatively constant level, we minimize a possible non-linearity of the interaction between the NMDA receptor and Ca^{2+} channel-mediated contribution to the integral postsynaptic calcium signal, thus maintaining the more uniform induction conditions. When LTP at the cortical input to the amygdala was compared (in a blinded fashion) in slices from control and from GRPR knockout mice, we found that LTP was significantly greater in knockout than in control mice (Figure 5B), with an average LTP of the EPSC to 2.02 ± 0.2 (n = 12 cells) and 1.33 ± 0.13 (n = 9 cells) of the baseline EPSC value, respectively. The difference in the amount of LTP measured over a 5 min period (between 35 and 40 min after pairing) between control and knockout mice was statistically significant (t test, t = 2.96, P < 0.01). Thus, the ablation of the *Grpr* gene disinhibits the pyramidal cells and makes the cortico-amygdala synapses more susceptible to LTP.

To obtain independent support for this conclusion, we measured the pairing-induced LTP in slices from wild-type mice in the presence of the bombesin antagonist. Under these conditions, LTP also was significantly enhanced (Figure 5D, control LTP: 1.42 ± 0.04, n = 5 cells; LTP with the antagonist: 1.92 ± 0.05, n = 7 cells; significant difference, t test, $t = 8.1$, $P < 0.0001$).

GRPR-Deficient Mice Have Enhanced and Persistent Long-Term Memory for Fear to Both Auditory and Contextual Cues

We first trained GRPR-deficient mice in Pavlovian cued and contextual fear conditioning, an amygdala-dependent task, which depends on the ability of the animal to learn and remember that auditory cue or context predict electric shock. During training, the level of overall freezing of knockout animals was not significantly different from wild-type littermate controls. For both groups, freezing was slightly increased within 30 s immediately after training (Figure $6A_1$).

When tested for amygdala-dependent tone fear con-

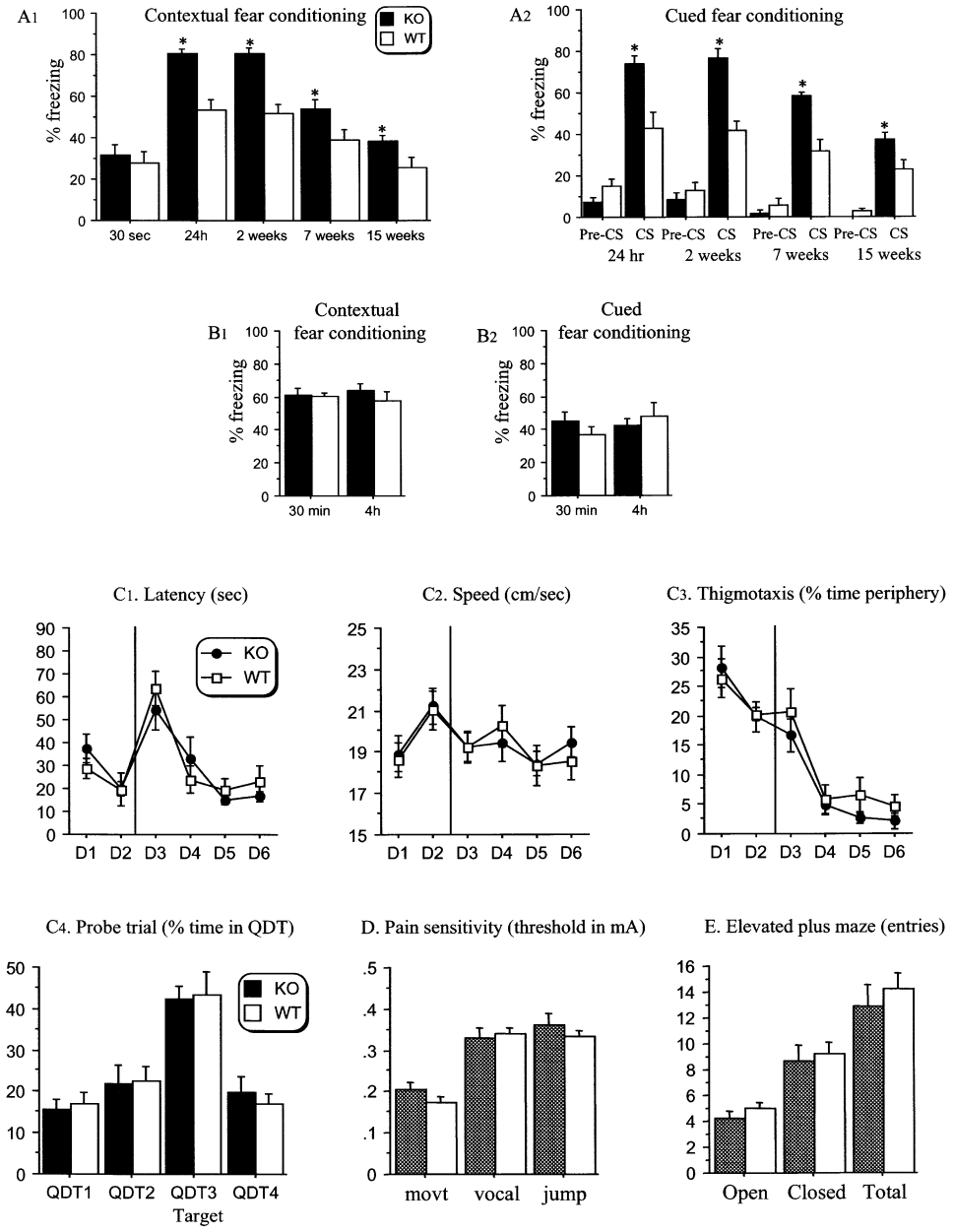


Figure 6. GRPR-Deficient Mice Have Enhanced and Resistant Long-Term But Not Short-Term Amygdala-Dependent Fear Memory

(A_1) Contextual fear conditioning. Significant difference in freezing responses between GRPR knockout mice (n = 9, solid bars) and wild-type (n = 9, open bars) mice was found at 24 hr, 2, 7, and 15 weeks after training.

(A_2) Cued fear conditioning. In response to the tone (CS), both groups showed an increase in freezing. However, this increase was significantly higher in GRPR knockout animals, although no difference was found between groups in the level of freezing before the onset of the tone (pre-CS).

(B_1) Contextual and (B_2) cued-fear conditioning assessed 30 min or 4 hr after training was normal in GRPR knockout mice. Water maze (C_{1-4}; wild-type, n = 9; knockout, n = 9). In this hippocampus-dependent memory task, both groups of mice showed a similar rate of learning as demonstrating by their equivalent latency (C_1) to reach the platform, whether it is during the visible (Day 1 and 2) or hidden platform version of the task (Day 3–6). They displayed the same swimming speed (C_2), and thigmotaxis (% of time spent at the periphery; C_3). They also showed equivalent performance in the probe trial (% of time spent in the different quadrant areas; C_4), which assessed the retention of spatially acquired information necessary to perform this task. GRPR knockout mice are no more sensitive or stressed than wild-type mice (D and E). Pain sensitivity thresholds (D). The intensity of shock required to elicit three reactions, movement (movt), vocalization (vocal), and jump, was assessed and data are presented as the mean ± SEM. No difference was found between groups (wild-type, n = 10; knockout, n = 8). Elevated plus maze assessing basal anxiety (E). No difference was found between GRPR (n = 18) and wild-type mice (n = 16) in the total number of entries, as in the number of entries in the closed or open arms.

ditioning, mice were placed in a new context 24 hr after training (Figure $6A_2$). Mice displayed an increase in freezing at the onset of the tone (CS; cued fear conditioning) as compared to the freezing prior (pre-CS) to the tone (Session effect, all $p < 0.01$). In addition, the ANOVA revealed a significant effect of genotype showing that GRPR knockout mice froze more than the wild-type mice at the presentation of the tone which had been associated previously with the electric shock (genotype effect: [$F(1,16) = 13.30$; $p = 0.002$]). Although freezing decreased with time in both groups of mice (Session effect; all $p < 0.01$), GRPR knockout mice produced a higher response to the tone in subsequent CS cued-testing sessions at 2, 7, and 15 weeks (Genotype effect; all $p < 0.05$).

Contextual fear conditioning is dependent both on the amygdala and the hippocampus. Here, mice were tested in the absence of cue in the same context 24 hr after training. Both mutant and wild-type mice exhibited higher level of freezing compared to immediately after the shock (Session effect, all $p < 0.0001$, Figure $6A_1$). This suggests that the mutant mice not only remembered the context where they received the shock the day before, but that they also developed with time a strong aversive response to this environment associated with a painful experience. The ANOVA revealed a significant effect of genotype ([$F(1,16) = 25.07$; $p = 0.0001$]) showing that both groups of mice froze differently in this context, with GRPR knockout mice showing a higher response as compared to their control littermates. Although freezing to context decreased in both groups of mice with time (Session effect, all $p < 0.0001$) suggesting similar rate of extinction, the observed increase in freezing in GRPR knockout mice was still present in subsequent testing sessions at 2, 7, and 15 weeks (Genotype effect, all $p < 0.05$).

We also analyzed mutant mice for short-term memory at 30 min and at 4 hr in independent groups. For both time points, there was no significant difference between mutant and wild-type mice in both contextual and cued fear conditioning (Figure 6B). Thus, the enhancement in learned fear observed in GRPR knockout mice is specific to long-term but not short-term memory.

To verify that the increase in freezing displayed by GRPR knockout mice in the fear-conditioning experiment was not due to an increased sensitivity to the shock, we performed a control experiment in which we administered electric shock of increasing intensity while recording the behavioral response exhibited by the mice (Harrel, 2001). There was no difference between groups in the intensity of shock required to elicit movement, vocalization, or jump (Figure 6D), indicating that an increase in freezing observed in the fear conditioning experiments was due to the learning process and not to a difference in pain sensitivity.

The Enhanced and Persistent Fear Is Learned and Not Secondary to Chronic Anxiety

To explore further these mice's tendency for innate (not learned) fear, we used the elevated plus maze where mice face a conflict between an innate aversion to the open arms of the maze and the motivation to explore this compartment (Ramboz et al., 1998). The ANOVA conducted on the number of entries in the open and closed arms and on the index of anxiety (time spent/entries in the open arms) did not reveal any significant effect of genotype (Figure 6E). Thus, in the elevated plus maze, the basal level of anxiety was similar in control and GRPR knockout mice.

Another way to assess anxiety in mice is a light-dark box test (Johansson et al., 2001). In this test, mice tend to avoid the light compartment and naturally prefer the dark one. Here again, we did not find any difference between groups in the number of entries as well as the total time spent in the lit compartment (data not shown). Thus, as with the elevated plus maze, the results from the light-dark box test suggest that the basal level of anxiety in GRPR knockout mice is similar to that of wild-type mice.

The GRPR Knockout Mice Show Normal Hippocampus-Dependent Spatial Memory

Because GRP is expressed in the lateral nucleus of the amygdala and specifically in its circuitry for learned fear and we have found that knockout of GRPR enhances amygdala-based learning, we were curious to know if we can use GRPR-deficient mice to dissociate amygdala-dependent from hippocampus-dependent learning. To determine whether GRPR is important for a purely hippocampus-based task, we turned to the Morris water maze, a task in which the amygdala is not involved. In this maze, an animal has to remember the position of a hidden escape platform in relationship to distal cues surrounding it in a circular pool (Malleret et al., 1999). During acquisition of the Morris water maze, mice from all groups showed a decrease in escape latency (Figure $6C_1$) across days, indicating learning of the platform position (all groups, $p < 0.0001$). They also showed a preference for the target quadrant during the probe trial performed on the last day of the experiment (Figure $6C_4$). We found no differences between groups in this task (no genotype effect), suggesting that the deletion of the GRPR does not enhance hippocampus-dependent learning that is independent of the amygdala, which is similar to the results of Wada and coworkers (Wada et al., 1997). These results support the notion that the amygdala is directly involved in learned fear (Fanselow and LeDoux, 1999) and that it does not merely modulate, emotionally, memories formed in other brain structures like the hippocampus.

Discussion

We have identified, characterized, and localized to a specific inhibitory neural circuit in the lateral nucleus of the amygdala a molecular signaling network important for learned fear. When this inhibitory molecular network is disrupted, mice show increased LTP in the lateral nucleus of the amygdala and an enhanced memory of learned fear as evident in both cued and contextual fear conditioning. There is a normal memory for hippocampus-based spatial task indicating that this network is specifically involved in the regulation of memory formation in the amygdala in response to danger signals. There also is no alteration in innate fear.

Experiments in humans and in experimental animals

over the last half a century indicate that the amygdala is involved in learned fear (Davis and Whalen, 2001; LeDoux, 2000). In the past 50 years, we have learned a fair amount about the anatomy and cell physiology underlying amygdala-based fear. For example, recent experiments have demonstrated that the mechanisms of LTP are recruited behaviorally at the synapses in the lateral nucleus of the amygdala during training for learned fear (Rogan et al., 1997; McKernan and Shinnick-Gallagher, 1997; Tsvetkov et al., 2002), thus providing direct support for the link between LTP and memory storage. By contrast, very little is known about the molecular mechanisms contributing to this form of fear. This is unfortunate because the neuronal pathways carrying sensory information for unimodal learned fear (the information carried by the CS) is much better specified than that for the sensory information for spatial learning as is the correlation between LTP and memory storage.

We therefore have isolated amygdala-enriched genes and then, using mouse genetics in combination with physiological and behavioral approaches studied the role of these genes in the memory for fear. Initially, we isolated two genes expressed in a glutamatergic principal neuron of the lateral nucleus of the amygdala. The first of these genes, *Op18/Stathmin*, is highly expressed both in the lateral nucleus of the amygdala and in the cerebral cortex with very little expression in the hippocampus. Op18/Stathmin is a phosphoprotein that binds tubulin dimers and destabilizes cellular microtubules (Belmont and Mitchison, 1996). It is a major substrate for protein kinase A and upon phosphorylation releases tubulin thus allowing polymerization of tubulin molecules. *Op18/Stathmin* mRNA levels are increased after lesions to the perforant pathway of the hippocampus, which together with the biochemical role of Op18/Stathmin protein suggests its involvement in synaptogenesis (Brauer et al., 2001).

The second gene, *Grp*, is uniquely localized in the lateral and accessory basal nuclei of the amygdala and in regions that send projections to it and which are essential for delivering information about CS to the amygdala during Pavlovian fear conditioning (LeDoux, 2000). In particular, our analysis showed that the *Grp* gene is expressed both in the areas specific to pathways delivering tone CS information and in the areas specific to pathways delivering contextual CS information. GRP is a 29 amino acid long mammalian homolog of the amphibian peptide bombesin (Kroog et al., 1995) and may serve as a cotransmitter with glutamate in pyramidal neurons in the rodent brain (Lee et al., 1999 and our present data). Our observation of the *Grp* gene expression pattern specific to the fear network of the amygdala finds support in the report that GRP concentration was increased in the central nucleus of the rat amygdala during both stress and feeding (Merali et al., 1998). GRPR is a $G\alpha q$ protein-coupled receptor and its downstream targets include protein kinase C (PKC-β) and phospholipase C as shown both in cultured mouse fibroblasts and rat hippocampal neurons (Hellmich et al., 1999; Lee et al., 1999). GRPR activation by GRP binding leads to intracellular release of Ca^{2+} and eventually to the activation of the MAPK pathway (Sharif et al., 1997).

We found that GRP is expressed in a group of glutamatergic principal neurons enriched in zinc. Interestingly, zinc-containing glutamatergic neurons constitute a specific network circuitry that includes the lateral nucleus of the amygdala and other components of the limbic system (reviewed in Frederickson et al., 2000). We next found, as did Lee and colleagues (1999), that GRPR is expressed in GABAergic interneurons. We also found that GRPR activation can significantly enhance the level of tonic GABA-mediated inhibition in the lateral nucleus. Recent pharmacological and genetic studies have shown that the establishment of a balance between glutamatergic excitatory and GABAergic inhibitory functions is critical for processing of information in the amygdala (Bast et al., 2001; Krezel et al., 2001). Based on these published data and our results, we suggest a model of GRP action in the amygdala during fear response; during excitation, the glutamatergic principal cells may release as a cotransmitter the excitatory peptide, GRP. Through the binding to GRPR on interneurons, GRP leads to GABA release. This may provide tonic, feedforward, or feedback inhibitory control of the processing of CS stimuli by principal cells (Figure 7, left image). Thus, this molecular signaling pathway provides a control which can regulate the balance between excitatory and inhibitory circuitries in the amygdala.

GRPR-Deficient Mice Show Both Enhanced LTP and Enhanced Memory Storage in Amygdala-Dependent Tasks

To test this model, we next examined the pyramidal neurons in the lateral nucleus of the amygdala of GRPR knockout animals and found that indeed they lack an inhibitory control normally provided by GRP in wild-type conditions (Figure 7, right image). As a result of lacking inhibition, there is an enhanced LTP in the cortico-amygdala pathway. In agreement with our genetic finding, previous pharmacological work has demonstrated that modulation of the level of GABA-mediated inhibition of the principal cells in the amygdala may determine how easily LTP is induced at the amygdala synapses (Krezel et al., 2001; Rammes et al., 2000).

Consistent with an enhancement of LTP, these animals also show enhanced freezing in both cued and contextual versions of amygdala-dependent fear conditioning task. Throughout all time points tested (the latest—15 weeks after training), mutant mice had higher freezing than normal mice. This may be due to faster fear memory retrieval in mutant mice because during testing mutants started freezing right after the tone was turned on but wild-types froze a few seconds later. The fading over time of the phenotype of GRPR knockout mice for fear conditioning might reflect the contribution of shock-induced sensitization in addition to the enhancement in learning. In contrast to long-term effects, we found that mutant mice have normal short-term memory when tested at 30 min and even 4 hr after training. This finding suggests the interesting possibility that GRP/GRPR signaling pathway modulates learned fear in a long-term specific manner and thus provides further support to the notion that LTP is implicated in the mechanisms of long-term memory. Importantly, GRPR-deficient mice showed normal memory in the Morris water maze, which is dependent on the hippocampus but not the amygdala. This finding is again consistent

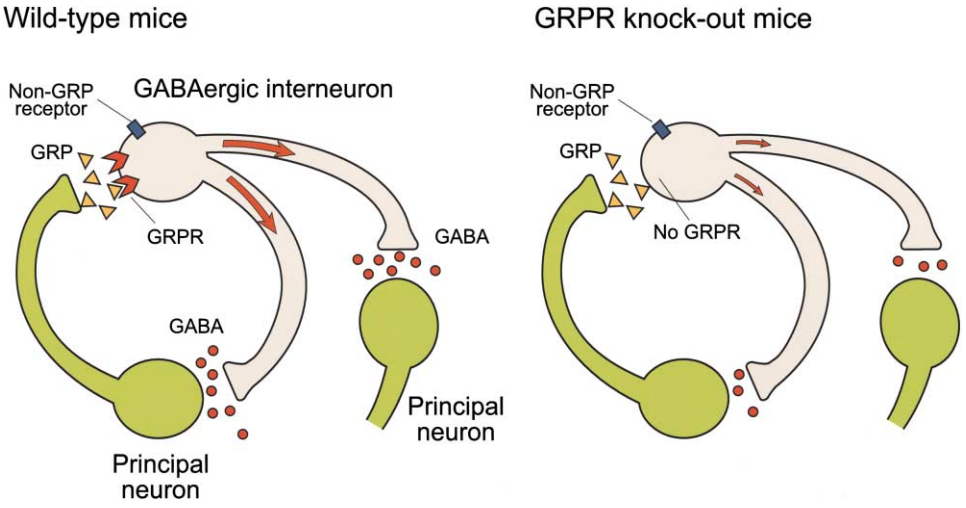


Figure 7. A Model for GRP-Dependent Negative Feedback to Principal Neurons in the Amygdala in Wild-Type and GRPR Knockout Mice

with fear circuitry-specific expression pattern of the *Grp* gene and allowed us to dissociate amygdala-based behavior from hippocampus-based behavior. Thus, we identified a network that is specifically involved in amygdala-dependent long-term memories for fear.

A Possible Role of GRP Pathway in Mental Disorders

The analysis of mice with decreased GABA function may have important clinical implications. Decreased levels of GABA have consistently been found in patients with depression, panic, and generalized anxiety disorders (Goddard et al., 2001) and some of the drugs currently used to treat panic and generalized anxiety disorders increase levels of GABA in the brain (Parent et al., 2002). We did not find any abnormalities in basal or in innate anxiety of GRPR knockout mice probably because we did not disrupt directly the biochemical machinery involved in the GABA production and utilization. Rather, we interfered with the GABA functions by disrupting a network that regulates GABA release. The reduction in GABA release in mutant mice seems to fine-tune the memory storage system so as to improve memory storage for fear. Perhaps, greater depleting GABA would lead to the opposite effects; it might decrease memory storage for fear and lead to high levels of anxiety similar to that described in mice mutant for GABA receptors (Low et al., 2000; McKernan et al., 2000). Since of all mental disorders anxiety disorders are those that can best be modeled in mice and other experimental animals (Bachevalier et al., 2001), it is likely that molecular insights in the biology of fear will prove to be broadly informative reagarding the genes important both for normal human fear and for anxiety states.

Indeed, recent studies have suggested the possible involvement of GRP and GRPR in mental disorders. *GRPR* is a candidate gene for autism; an X;8 translocation has been found that disrupted the first intron of the *GRPR* gene in an autistic female patient (Ishikawa-Brush et al., 1997). Importantly, genetic studies in autistic patients have pinpointed chromosomal abnormalities in the 15q11-q13, a region where the *GABRB3* gene is located, which codes for the β3 subunit of the γ-aminobutyric acid $(GABA)_A$ receptor (Cook et al., 1998). Moreover, recent behavioral, anatomical, and neuroimaging studies suggest that one of the critical loci for autism resides in the amygdala (Baron-Cohen et al., 2000).

Taken altogether, these observations demonstrate the importance of determining molecular substrates of amygdala-dependent memory processes and identify the components of GRP/GRPR molecular network as a clear target for potential treatment of anxiety disorders.

Experimental Procedures

Animals

GRPR knockout mice were described before and were found grossly normal (Hampton et al., 1998). Mice used for the study were backcrossed to N10 or more to C57BL/6J strain.

Differential Screening and In Situ Hybridization/Immunohistochemistry

Differential Screening

Amygdala cells were acutely dissociated as described (Yu and Shinnick-Gallagher, 1997). Cells morphologically resembling pyramidal neurons were identified under low magnification Nikon microscope and individually transferred to PCR tubes containing lysis buffer. cDNA libraries were synthesized as described (Dulac and Axel, 1995). Five thousand clones were differentially screened with the amygdala and CA1 single cell cDNA probes. Amygdala probes for the differential screening were enriched by two rounds of subtraction of representational difference analysis (Hubank and Schatz, 1994) against the CA1 cDNA.

In Situ Hybridization/Immunohistochemistry

Coronal sections from fresh-frozen mouse brains were cut 20 μm thick and hybridized according to the published protocol with modifications (Schaeren-Wiemers and Gerfin-Moser, 1993). For dual fluorescent in situ hybridization and immunohistochemistry, digoxigenin-labeled RNA was first detected using tyramide-based TSA Direct Fluorescein Kit (Perkin Elmer). Then, sections were incubated with rabbit antibody recognizing glutamic acid decarboxylase (Chemicon) and detected using Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch).

Electrophysiology

Amygdala slices (250–300 μm) were prepared from 3–5 week old control and GRPR knockout mice (littermates) with a vibratome.

Slices were continuously superfused in solution containing (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.25 NaH_2PO_4, 26.0 $NaHCO_3$, 10 glucose, and equilibrated with 95% O_2 and 5% CO_2 [pH 7.3–7.4] at room temperature. Whole-cell recordings of evoked compound EPSCs or spontaneous GABA-mediated IPSCs were obtained from pyramidal cells in the lateral amygdala under visual guidance (DIC/infrared optics) with an EPC-9 amplifier and Pulse v8.09 software (HEKA Elektronik). Compound EPSCs were evoked by stimulation of the fibers in the external capsule at 0.05 Hz with a concentric stimulating electrode consisting of a patch pipette (10 μm tip diameter) that was coated with silver paint (Bolshakov et al., 1997). The two leads of the stimulus isolation unit (ISO-Flex, Master-8 stimulator, AMPI, Jerusalem, Israel) were connected to the inside of the pipette and the external silver coat. The stimulating pipette was positioned to activate the cortical input to the lateral amygdala. To elicit the evoked $GABA_A$ IPSCs in the presence of CNQX (20 μM) and D-APV (50 μM) in the bath, the stimulation electrode was placed within the lateral nucleus of the amygdala. The patch electrodes (3–5 MΩ resistance) contained (in mM): 120 KCl, 5 NaCl, 1 $MgCl_2$, 0.2 EGTA, 10 HEPES, 2 MgATP, and 0.1 NaGTP (adjusted to pH 7.2 with KOH). In LTP experiments, 120 mM K-gluconate was used instead of KCl. To examine the voltage dependence of the evoked $GABA_A$ receptor IPSCs, cesium was substituted for potassium in the pipette solution. Series resistance was monitored throughout experiment and was in a range of 10–20 MΩ. Currents were filtered at 1 kHz and digitized at 5 kHz. The holding potential was -70 mV. In all LTP experiments, the stimulus intensity was adjusted to produce synaptic responses with an amplitude which constitutes $\sim$20%–25% of maximum amplitude EPSC. Since we controlled for the size of the baseline EPSC, the induction conditions were identical for both LTP groups (control and knockout mice). The EPSC amplitudes were measured as the difference between the mean current during a prestimulus baseline and the mean current over a 2 ms-window at the peak of the response. For induction of LTP, 80 presynaptic stimuli were delivered at 2 Hz to the external capsule fibers while the lateral nucleus of the amygdala neuron was held at $+30$ mV for the duration of the LTP-inducing presynaptic stimulation. Summary LTP graphs were constructed by normalizing data in 60 s epochs to the mean value of the baseline EPSC.

The spontaneous IPSCs were recorded on videotape for off line analysis in the presence of 20 μM CNQX. Data were analyzed with the Mini Analysis Program v5.2.4 (Synaptosoft Inc., Decatur, GA; Bolshakov et al., 2000).

Behavior

For all behavioral tasks, mutant and control littermates (males, 3 months old) were used. Statistical analyses used ANOVAs with genotype as the between subject factor, and session (fear conditioning experiment), day, area (quadrant or platform in the Morris water maze), or zone (elevated plus maze and light-dark box) as within subject factors. Mean $\pm$ SEM are presented. The experimenter was blind to the genotype in all studies.

Fear conditioning experiments were done as described (Bourtchouladze et al., 1998). On the training day, the mouse was placed in the conditioning chamber (Med Associates) for 2 min before the onset of CS, a tone, which lasted for 30 s at 2800 Hz, 85 dB. The last 2 s of the CS was paired with US, 0.7 mA of continuous foot shock. After an additional 30 s in the chamber, the mouse was returned to its home cage. Conditioning was assessed for 3 consecutive min in the chamber in which the mice were trained by scoring freezing behavior, which was defined as the complete lack of movement, in intervals of 5 s. Mice (wild-type, n = 9; knockout, n = 9) were tested immediately after training and at 24 hr, 2, 7, and 15 weeks after training. For each time point, testing occurred first in the context in which mice were trained (contextual fear conditioning). Three hours after each contextual testing session, mice were placed in a novel environment (cued fear conditioning) in which the tone (120 s) that has been presented during training was given after a 1 min habituation period (pre-CS).

Pain Sensitivity Tests

Response to foot shocks was assessed with naïve mice (wild-type, n = 10; knockout, n = 8) as described (Harrel, 2001). The intensity of shock required to elicit running, vocalization, and a jump was determined for each mouse by delivering a 1 s shock every 30 s starting at 0.08 mA and increasing the shock 0.02 mA each time. Testing was stopped after all behaviors had been noted.

Anxiety Tests

We performed two different tasks to assess basal anxiety level in naïve mice.

Elevated Plus Maze

The elevated plus maze consisted of a center platform and four arms placed 50 cm above the floor (Ramboz et al., 1998). Two arms were enclosed within walls and the other two (open) had low rims. Naïve mice (wild-type, n = 18; knockout, n = 16) were placed in the center and their behavior was recorded for 5 min with a camera located above the maze. Time spent (in seconds, s) and entries in the different compartments (closed and open arms, center) were assessed.

Dark-Light Box

For the dark-light box test, mice (wild-type, n = 10; knockout, n = 9) were placed in the dark compartment (head facing the wall) and observed for 5 min (Johansson et al., 2001). Time spent in and entries into the lit compartment were recorded.

Water Maze

The task was performed as previously described (Malleret et al., 1999) with two training phases: 2 days with a visible platform followed by 4 days (spatial phase) with a hidden platform in the training quadrant (wild-type, n = 9; knockout, n = 9). For each phase, four trials, 120 s maximum and 15 min ITI (inter-trial interval) were given daily. Probe trials (60 s), during which the platform was removed, were performed to assess retention of the previously acquired information.

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References

Bachevalier, J., Malkova, L., and Mishkin, M. (2001). Effects of selective neonatal temporal lobe lesions on socioemotional behavior in infant rhesus monkeys (Macaca mulatta). Behav. Neurosci. *115*, 545–559.

Baron-Cohen, S., Ring, H.A., Bullmore, E.T., Wheelwright, S., Ashwin, C., and Williams, S.C. (2000). The amygdala theory of autism. Neurosci. Biobehav. Rev. *24*, 355–364.

Bast, T., Zhang, W.N., and Feldon, J. (2001). The ventral hippocampus and fear conditioning in rats. Different anterograde amnesias of fear after tetrodotoxin inactivation and infusion of the GABA(A) agonist muscimol. Exp. Brain Res. *139*, 39–52.

Belmont, L.D., and Mitchison, T.J. (1996). Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. Cell *84*, 623–631.

Bolshakov, V.Y., Golan, H., Kandel, E.R., and Siegelbaum, S.A. (1997). Recruitment of new sites of synaptic transmission during the cAMP-dependent late phase of LTP at CA3–CA1 synapses in the hippocampus. Neuron *19*, 635–651.

Bolshakov, V.Y., Carboni, L., Cobb, M.H., Siegelbaum, S.A., and Belardetti, F. (2000). Dual MAP kinase pathways mediate opposing forms of long-term plasticity at CA3–CA1 synapses. Nat. Neurosci. *3*, 1107–1112.

Bourtchouladze, R., Abel, T., Berman, N., Gordon, R., Lapidus, K., and Kandel, E.R. (1998). Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. Learn. Mem. *5*, 365–374.

Brauer, A.U., Savaskan, N.E., Plaschke, M., Ninnemann, O., and Nitsch, R. (2001). Perforant path lesion induces up-regulation of stathmin messenger RNA, but not SCG10 messenger RNA, in the adult rat hippocampus. Neuroscience *102*, 515–526.

Cook, E.H., Jr., Courchesne, R.Y., Cox, N.J., Lord, C., Gonen, D., Guter, S.J., Lincoln, A., Nix, K., Haas, R., Leventhal, B.L., and Courchesne, E. (1998). Linkage-disequilibrium mapping of autistic disorder, with 15q11–13 markers. Am. J. Hum. Genet. *62*, 1077–1083.

Davis, M., and Whalen, P.J. (2001). The amygdala: vigilance and emotion. Mol. Psychiatry *6*, 13–34.

Dulac, C., and Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals. Cell *83*, 195–206.

Fanselow, M.S., and LeDoux, J.E. (1999). Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala. Neuron *23*, 229–232.

Frederickson, C.J., Suh, S.W., Silva, D., and Thompson, R.B. (2000). Importance of zinc in the central nervous system: the zinc-containing neuron. J. Nutr. *130*, 1471S–1483S.

Goddard, A.W., Mason, G.F., Almai, A., Rothman, D.L., Behar, K.L., Petroff, O.A., Charney, D.S., and Krystal, J.H. (2001). Reductions in occipital cortex GABA levels in panic disorder detected with 1h-magnetic resonance spectroscopy. Arch. Gen. Psychiatry *58*, 556–561.

Hampton, L.L., Ladenheim, E.E., Akeson, M., Way, J.M., Weber, H.C., Sutliff, V.E., Jensen, R.T., Wine, L.J., Arnheiter, H., and Battey, J.F. (1998). Loss of bombesin-induced feeding suppression in gastrin-releasing peptide receptor-deficient mice. Proc. Natl. Acad. Sci. USA *95*, 3188–3192.

Harrel, A.V., Caldwell, K.K., and Allan, A.M. (2001). Transgenic mice over-expressing the 5–HT3 receptor have enhanced learning in latent inhibition and contextual fear conditioning paradigms. Paper presented at Abstracts, Society for Neuroscience, 31st Annual Meeting, San Diego, California.

Hellmich, M.R., Ives, K.L., Udupi, V., Soloff, M.S., Greeley, G.H., Jr., Christensen, B.N., and Townsend, C.M., Jr. (1999). Multiple protein kinase pathways are involved in gastrin-releasing peptide receptor-regulated secretion. J. Biol. Chem. *274*, 23901–23909.

Huang, Y.Y., and Kandel, E.R. (1998). Postsynaptic induction and PKA-dependent expression of LTP in the lateral amygdala. Neuron *21*, 169–178.

Hubank, M., and Schatz, D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res. *22*, 5640–5648.

Ishikawa-Brush, Y., Powell, J.F., Bolton, P., Miller, A.P., Francis, F., Willard, H.F., Lehrach, H., and Monaco, A.P. (1997). Autism and multiple exostoses associated with an X;8 translocation occurring within the GRPR gene and 3′ to the SDC2 gene. Hum. Mol. Genet. *6*, 1241–1250.

Johansson, B., Halldner, L., Dunwiddie, T.V., Masino, S.A., Poelchen, W., Gimenez-Llort, L., Escorihuela, R.M., Fernandez-Teruel, A., Wiesenfeld-Hallin, Z., Xu, X.J., et al. (2001). Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. Proc. Natl. Acad. Sci. USA *98*, 9407–9412.

Kapp, B.S., Whalen, P.G., Supple, W.F., and Pascoe, J.P. (1992). Amygdaloid contributions to conditioned arousal and sensory information processing. In The Amygdala: Neurobiological Aspects of Emotion, Memory, and Mental Dysfunction, J.P. Aggleton, ed. (New York: Wiley-Liss), pp. 229–254.

Krezel, W., Dupont, S., Krust, A., Chambon, P., and Chapman, P.F. (2001). Increased anxiety and synaptic plasticity in estrogen receptor β-deficient mice. Proc. Natl. Acad. Sci. USA *98*, 12278–12282.

Kroog, G.S., Jensen, R.T., and Battey, J.F. (1995). Mammalian bombesin receptors. Med. Res. Rev. *15*, 389–417.

LeDoux, J.E. (2000). Emotion circuits in the brain. Annu. Rev. Neurosci. *23*, 155–184.

Lee, K., Dixon, A.K., Gonzalez, I., Stevens, E.B., McNulty, S., Oles, R., Richardson, P.J., Pinnock, R.D., and Singh, L. (1999). Bombesin-like peptides depolarize rat hippocampal interneurones through interaction with subtype 2 bombesin receptors. J. Physiol. *518*, 791–802.

Low, K., Crestani, F., Keist, R., Benke, D., Brunig, I., Benson, J.A., Fritschy, J.M., Rulicke, T., Bluethmann, H., Mohler, H., and Rudolph, U. (2000). Molecular and neuronal substrate for the selective attenuation of anxiety. Science *290*, 131–134.

Mahanty, N.K., and Sah, P. (1998). Calcium-permeable AMPA receptors mediate long-term potentiation in interneurons in the amygdala. Nature *394*, 683–687.

Malleret, G., Hen, R., Guillou, J.L., Segu, L., and Buhot, M.C. (1999). 5–HT1B receptor knock-out mice exhibit increased exploratory activity and enhanced spatial memory performance in the Morris water maze. J. Neurosci. *19*, 6157–6168.

McKernan, M.G., and Shinnick-Gallagher, P. (1997). Fear conditioning induces a lasting potentiation of synaptic currents in vitro. Nature *390*, 607–611.

McKernan, R.M., Rosahl, T.W., Reynolds, D.S., Sur, C., Wafford, K.A., Atack, J.R., Farrar, S., Myers, J., Cook, G., Ferris, P., et al. (2000). Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha1 subtype. Nat. Neurosci. *3*, 587–592.

Merali, Z., McIntosh, J., Kent, P., Michaud, D., and Anisman, H. (1998). Aversive and appetitive events evoke the release of corticotropin-releasing hormone and bombesin-like peptides at the central nucleus of the amygdala. J. Neurosci. *18*, 4758–4766.

Mermelstein, P.G., Bito, H., Deisseroth, K., and Tsien, R.W. (2000). Critical dependence of cAMP response element-binding protein phosphorylation on L-type calcium channels supports a selective response to EPSPs in preference to action potentials. J. Neurosci. *20*, 266–273.

Parent, M.B., Master, S., Kashlub, S., and Baker, G.B. (2002). Effects of the antidepressant/antipanic drug phenelzine and its putative metabolite phenylethylidenehydrazine on extracellular γ-aminobutyric acid levels in the striatum. Biochem. Pharmacol. *63*, 57–64.

Pitkanen, A., Savander, V., and LeDoux, J.E. (1997). Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. Trends Neurosci. *20*, 517–523.

Ramboz, S., Oosting, R., Amara, D.A., Kung, H.F., Blier, P., Mendelsohn, M., Mann, J.J., Brunner, D., and Hen, R. (1998). Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. Proc. Natl. Acad. Sci. USA *95*, 14476–14481.

Rammes, G., Steckler, T., Kresse, A., Schutz, G., Zieglgansberger, W., and Lutz, B. (2000). Synaptic plasticity in the basolateral amygdala in transgenic mice expressing dominant-negative cAMP response element-binding protein (CREB) in forebrain. Eur. J. Neurosci. *12*, 2534–2546.

Rogan, M.T., Staubli, U.V., and LeDoux, J.E. (1997). Fear conditioning induces associative long-term potentiation in the amygdala. Nature *390*, 604–607.

Romanski, L.M., and LeDoux, J.E. (1992). Equipotentiality of thalamo-amygdala and thalamo-cortico-amygdala circuits in auditory fear conditioning. J. Neurosci. *12*, 4501–4509.

Schaeren-Wiemers, N., and Gerfin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. Histochemistry *100*, 431–440.

Sharif, T.R., Luo, W., and Sharif, M. (1997). Functional expression of bombesin receptor in most adult and pediatric human glioblastoma cell lines; role in mitogenesis and in stimulating the mitogen-

activated protein kinase pathway. Mol. Cell. Endocrinol. *130*, 119–130.

Steele, P.M., and Mauk, M.D. (1999). Inhibitory control of LTP and LTD: stability of synapse strength. J. Neurophysiol. *81*, 1559–1566.

Trepel, C., and Racine, R.J. (2000). GABAergic modulation of neocortical long-term potentiation in the freely moving rat. Synapse *35*, 120–128.

Tsvetkov, E., Carlezon, W.A., Benes, F.M., Kandel, E.R., and Bolshakov, V.Y. (2002). Fear conditioning occludes LTP-induced presynaptic enhancement of synaptic transmission in the cortical pathway to the lateral amygdala. Neuron *34*, 289–300.

Wada, E., Watase, K., Yamada, K., Ogura, H., Yamano, M., Inomata, Y., Eguchi, J., Yamamoto, K., Sunday, M.E., Maeno, H., et al. (1997). Generation and characterization of mice lacking gastrin-releasing peptide receptor. Biochem. Biophys. Res. Commun. *239*, 28–33.

Wang, C., Wilson, W.A., and Moore, S.D. (2001). Role of NMDA, non-NMDA, and GABA receptors in signal propagation in the amygdala formation. J. Neurophysiol. *86*, 1422–1429.

Weisskopf, M.G., and LeDoux, J.E. (1999). Distinct populations of NMDA receptors at subcortical and cortical inputs to principal cells of the lateral amygdala. J. Neurophysiol. *81*, 930–934.

Weisskopf, M.G., Bauer, E.P., and LeDoux, J.E. (1999). L-type voltage-gated calcium channels mediate NMDA-independent associative long-term potentiation at thalamic input synapses to the amygdala. J. Neurosci. *19*, 10512–10519.

Woodson, W., Farb, C.R., and Ledoux, J.E. (2000). Afferents from the auditory thalamus synapse on inhibitory interneurons in the lateral nucleus of the amygdala. Synapse *38*, 124–137.

Yu, B., and Shinnick-Gallagher, P. (1997). Dihydropyridine- and neurotoxin-sensitive and -insensitive calcium currents in acutely dissociated neurons of the rat central amygdala. J. Neurophysiol. *77*, 690–701.